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The retinoblastoma protein, RB, is required for gastrointestinal endocrine cells to exit the cell cycle, but not for hormone expression

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Abstract

Important functions of the RB family proteins include inhibition of cell cycle progression and regulation of terminal differentiation. We have examined the role of RB and the related protein, p107, in regulating cell cycle activity and differentiation of gastrointestinal endocrine cells, a relatively quiescent cell population, by conditionally disrupting the *RB* gene in neurogenin3 (Ngn3)-expressing cells in both $p107^{+/+}$ and $p107^{-/-}$ mice. Endocrine cells in the small intestine, colon, pancreas, and stomach were present in normal numbers in RB and RB–p107 mutants except for an increase in serotonin cells and decrease in ghrelin cells in the antral stomach. Deletion of RB resulted in a dramatic increase in proliferating serotonin cells in the antral stomach and intestine, whereas other enteroendocrine cell types exhibited much lower cell cycle activity or remained quiescent. The related p107 protein appears dispensable for enteroendocrine differentiation and does not functionally compensate for the loss of RB. Our results suggest that RB is required for enteroendocrine cells, particularly serotonin cells, to undergo cell cycle arrest as they terminally differentiate. RB has relatively subtle effects on enteroendocrine cell differentiation and is not required for the expression of the normal repertoire of hormones in the gastrointestinal tract.

Keywords: Retinoblastoma protein; Enteroendocrine cell; NeuroD; Neurogenin 3

Introduction

The mammalian gastrointestinal epithelium undergoes perpetual self-renewal through carefully controlled cell proliferation, differentiation, and apoptosis to maintain itself throughout life. Different gastrointestinal organs exhibit significant differences in their rates of proliferation and turnover. The intestinal epithelium has the most rapid turnover rate among the gastrointestinal organs whereas the stomach and pancreas turnover more slowly.

Endocrine cells represent a relatively small fraction of the total gastrointestinal epithelium. In general, the self-renewal rates for endocrine cells reflect their organ of origin. The intestinal enteroendocrine cells undergo self-renewal every 4–5 days from a large reservoir of pluripotent cells in crypts, which give rise to a rapidly proliferating transit amplifying population of immature cells in the lower crypts that give rise to precursors for each endocrine cell type. These immature cells exit the cell cycle as they migrate up the crypt–villus axis during differentiation (Cheng and Leblond, 1974). In contrast, gastric enteroendocrine cells turn over more slowly than their intestinal counterparts with an estimated half-life of 10–15 days and turnover time of 100 days (Fujimoto et al., 1980; Karam and Leblond, 1993). The topography of gastric endocrine cells is also distinct from the intestine. Multipotent endocrine precursors reside in a proliferating zone in the midportion, the isthmus, of gastric glands. As cells mature, they migrate away from the isthmus towards either the base of the gland or the lumen, stop dividing, and differentiate. Unlike the stomach, the pancreatic islet cells turn over very slowly in adult animals with a half-life of approximately 47 days and turnover time of 250–520 days (Cameron, 1970; Magami et al., 2002; Tsubouchi et

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al., 1987). The existence of multipotent cells in adult islets is somewhat controversial with one study suggesting that islet cells are slowly replaced by division of existing differentiated cells rather than multipotent cells (Dor et al., 2004).

The retinoblastoma protein (RB) and its related "pocket" proteins p107 and p130 have been implicated in coordinating cell cycle activity with cellular differentiation (Sherr, 1996; Weinberg, 1995). RB functions to restrict expression of S phase genes by negatively regulating the activity of the transcription factor E2F. RB may also promote terminal differentiation by interacting with tissue-specific transcription factors (Lipinski and Jacks, 1999) or by its recruitment of histone deacetylases (Dyson, 1998). The RB related proteins, p107 and p130, also may have a role in cell cycle regulation and differentiation that in part overlaps the function of RB (Mulligan et al., 1998; Sage et al., 2003).

The temporal–spatial coordination of cell cycle exit with terminal differentiation in the gastrointestinal tract suggests a potential role for RB family proteins in the regulation of epithelial homeostasis. A recent report examined the role of RB and its related proteins in the regulation of epithelial homeostasis in the distal small intestine by conditionally deleting RB in mice under control of an intestine-specific *Fabpl-cre* transgene. Loss of RB, p107, or p130 alone had little effect whereas loss of RB plus either p130 or p107 resulted in mild chronic intestinal hyperplasia. Goblet cells and enterocytes in double mutant mice showed defects in cell maturation. Paneth cells and enteroendocrine cells expressing the general marker, chromogranin A, in the distal small intestine appeared to differentiate normally in RB–p130 mutants (Haigis et al., 2006). However, the effects on individual endocrine lineages were not examined in this study. The role of RB proteins in coordinating differentiation of specific lineages in other gastrointestinal organs, including the stomach, pancreas and colon, remains to be elucidated.

Differentiation of enteroendocrine cells is initiated by transient expression of the basic helix loop helix (bHLH) transcription factor, *Neurogenin 3* (*Ngn3*) in immature, proliferating cells in crypts (Jenny et al., 2002; Lee et al., 2002; Mutoh et al., 1997). As cells further differentiate, a second bHLH protein, NeuroD is required for the expression of at least two hormones, secretin and cholecystokinin (Mutoh et al., 1997; Naya et al., 1997). In addition to its effects on hormone expression, NeuroD appears to induce cell cycle arrest in proliferating cells (Mutoh et al., 1998). Relatively little is known about how enteroendocrine differentiation in the stomach is coordinated with loss of cell proliferation.

In the present work, we have examined the role of RB in cell cycle control and differentiation of the major endocrine cell types of the small intestine, colon, stomach and pancreas by conditionally deleting RB in cells expressing Ngn3. Although, RB appears to contribute to cell cycle exit of maturing endocrine cells, our results suggest that RB is not required for expression of hormonal markers characteristic of differentiated gastrointestinal endocrine cells. Compared to other endocrine lineages, an unusually large fraction of serotonin-expressing cells continues to proliferate in the absence of RB.

Results

Differentiation of endocrine cells in the intestine and colon occurs independently of RB and P107

To determine the potential role of RB in differentiation of enteroendocrine cells from their precursors, we conditionally deleted the *RB* gene in $Ngn3^{+}$ cells. We crossed $RB^{Flox/-}$ mice, with one null *RB* allele and one allele with loxP sites flanking exon 19 ($RB^{Flox/-}$) to mice expressing Cre recombinase under control of the *Ngn3* gene (Schonhoff et al., 2004). Because increased expression of the related protein, p107 often compensates for the loss of RB, the *Ngn3-Cre;*$RB^{Flox/-}$ mice were further crossed to a p107 null background.

Ngn3-Cre;$RB^{Flox/-}$ mice and *Ngn3-Cre;*$RB^{Flox/-}$*;*$p107^{-/-}$ mice were healthy and born at the expected frequency. The morphology of the gastrointestinal tract appeared to be histologically normal. To determine whether RB was required for the differentiation of enteroendocrine cells from Ngn3-expressing precursors, we examined the intestine and colon for expression of chromogranin A (ChgA), a marker that is expressed in most enteroendocrine cells. The number and distribution of ChgA stained cells in the mutant mice was comparable to that of the wild type (Figs. 1A, C, D). RB/p107 mutants showed no change in the number of small intestinal endocrine cells and appeared identical to RB single mutants, suggesting that p107 did not mask the effects of deleting RB. Likewise, the deletion of both RB and p107 showed no effect on the number of cells differentiating into individual endocrine cell lineages (Fig. 1B), indicating that endocrine cell specification in the intestine and colon does not require either protein.

To determine whether RB was required for differentiating endocrine cells to exit the cell cycle, we examined enteroendocrine cells in *Ngn3-Cre;*$RB^{Flox/-}$ mice for the expression of PCNA, a marker of proliferating cells. In wild type mice, most enteroendocrine cells appear quiescent. However, we identified approximately 10% of substance P-expressing enteroendocrine cells that stained for PCNA (Table 1), suggesting that substance P expression occurs in relatively immature cells versus other hormones. This observation is consistent with previous work identifying rapid BrdU uptake in substance P-expressing cells in small intestinal crypts but not with serotonin or secretin cells (Aiken and Roth, 1992). In *Ngn3-Cre;*$RB^{Flox/-}$ mice, up to 30% of serotonin cells in the small intestine and 16% of serotonin cells in the colon stained for PCNA. The cell cycle activity of serotonin cells following RB deletion noted here is similar to our recent observation that approximately 35% of serotonin cells stained for Ki67 following widespread deletion of the *RB* gene in the intestinal epithelium (Yang and Hinds, 2007). Further examination of major intestinal endocrine cell lineages revealed that serotonin cells represented the major population of proliferating enteroendocrine cells (Table 1). Most other enteroendocrine cells showed much smaller increases in cell cycle activity. Of note, duodenal ghrelin cells appear to be relatively quiescent and do not depend on RB for cell cycle withdrawal.

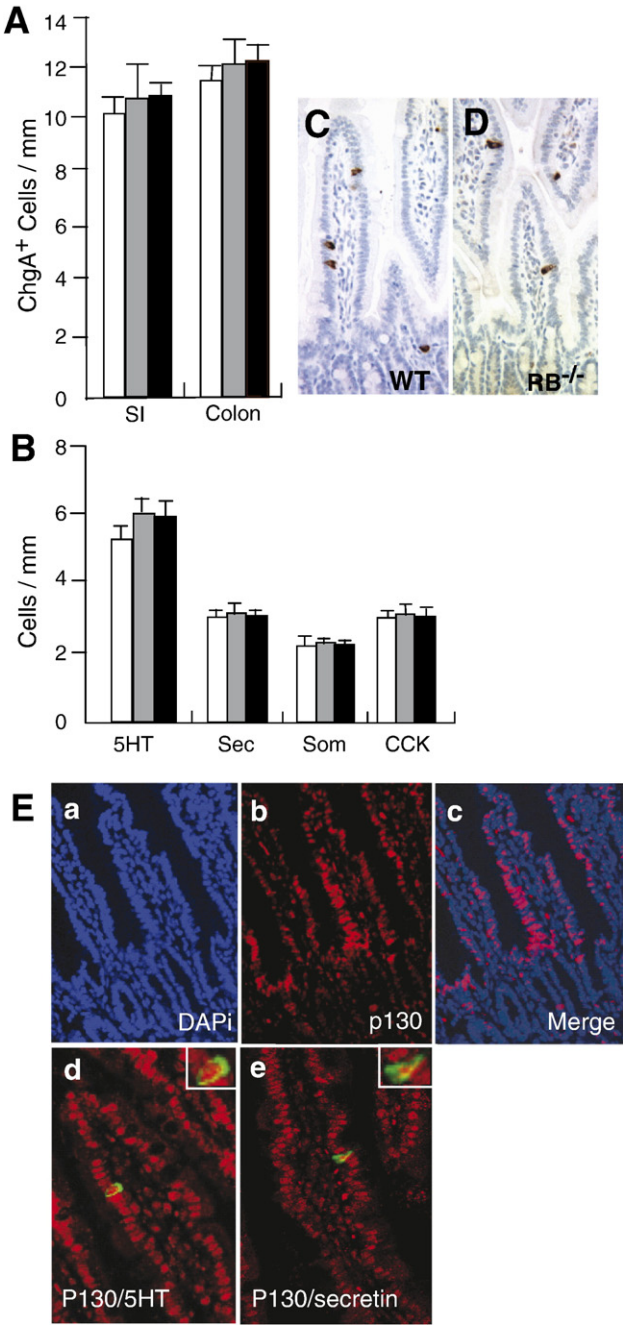

Fig. 1. Enteroendocrine cells in RB or RB/p107 mutant mice. (A, B) Morphometric analysis of intestinal endocrine cells from wild type (white bars), *Ngn3-Cre;RB$^{Flox/-}$* (*gray bars*) and *Ngn3-Cre;RB$^{Flox/-}$;P107$^{-/-}$* mice (black bars). (A) Chromogranin A (*ChgA*)-stained cells. (B) Numbers of serotonin, secretin, somatostatin, and cholecystokinin stained cells. Results were expressed as mean number of stained cells ± SEM per mm length of the intestine and colon viewed in longitudinal sections. (C, D) Chromogranin A-immunostained intestinal sections from *Ngn3-Cre;RB$^{Flox/-}$* mice (D) and wild type mice (C). Abbreviations: SI, small intestine; 5HT, serotonin; Sec, secretin; Som, somatostatin; CCK, cholecystokinin. (E) Confocal images of small intestine from *Ngn3-Cre;RB$^{Flox/-}$* mouse double stained for DAPI: (a) p130, (b) merge, (c) shown at low magnification. (d, e) RB mutant small intestine showing immunofluorescent staining for p130 in red and serotonin (d) or secretin (e) in *green*. Insets show a single serotonin or secretin cell with nuclear staining for p130 at high magnification.

Table 1
Cell cycle activity in intestinal endocrine cells

Cell type	% PCNA$^+$ cells	
	RB$^{-/-}$	WT
Small intestine		
Serotonin	31±4	0±0
Substance P	20±5	10±2
Secretin	3±3	0±0
CCK	3±1	0±0
GIP	4±2	0±0
Ghrelin	0±0	0±0
Large intestine		
Serotonin	16±3	0±0
PYY	0±0	0±0

Three animals per genotype were analyzed. At least 500 cells were counted for each lineage. Results expressed as percent cells staining for PCNA ± SEM. The difference between serotonin cells and other intestinal endocrine cells was significant with a P value <0.01.

The majority of PCNA labeled cells were located in the upper crypt or lower villus, implying delayed cell cycle exit as these cells migrate up the crypt–villus axis during differentiation. Deletion of RB had no effect on the number of caspase 3-positive cells suggesting that apoptosis was unaffected (not shown). Together, these results suggest RB expression contributes to cell cycle withdrawal of enteroendocrine cells as they mature. Examination of enteroendocrine cells of p107 null mice was identical to wild type mice. RB/p107 double mutant mice showed identical changes to the RB mutant mice, indicating that p107 does not compensate for the loss of RB. Thus cell cycle withdrawal of differentiating enteroendocrine cells appears to be specific for RB.

Examination of RB/p130 double mutants in an earlier study suggested that p130, appears to partially compensate for the loss of RB in the intestine (Haigis et al., 2006). To determine whether p130 might functionally compensate for the loss of RB in enteroendocrine cells that are minimally affected by the loss of RB but not serotonin cells, we examined whether serotonin and secretin cells in RB null mice express p130. Immunofluorescent staining revealed that p130 was predominantly expressed in villi but not crypts of the small intestine as previously described (Haigis et al., 2006) (Fig. 1E, a–c). Nearly all serotonin and secretin cells expressed p130 despite significant differences in their cell cycle activity in RB mutants (Fig. 1E, d, e). Thus it is unlikely that differences in p130 expression account for relatively high cell cycle activity of serotonin cells versus other enteroendocrine cell types in the absence of RB.

We previously showed that the basic helix loop helix protein, NeuroD, was necessary for inducing cell cycle arrest of enteroendocrine cells in addition to its effects on hormone gene transcription (Mutoh et al., 1998). We examined the small intestine of *Ngn3-Cre;RB$^{Flox/-}$* mice to determine whether the increased cell cycle activity resulted from the loss of NeuroD expression following deletion of RB. Triple immunofluorescent staining for NeuroD, PCNA, and either serotonin or secretin revealed that all PCNA-expressing secretin and

serotonin cells continued to express NeuroD (Figs. 2A, B), indicating that cell cycle exit induced by NeuroD may in part be mediated by RB.

Recent evidence suggests that RB may directly interact with NeuroD to form a bridging complex with the orphan nuclear receptor, NGF1B, on the pro-opiomelanocortin (POMC) promoter to potentiate POMC transcription in corticotrophs (Batsche et al., 2005). We examined the effects of RB on NeuroD-dependent transcription of the secretin gene as previously described (Mutoh et al., 1998) in C33A cells, a cell line that does not express functional RB or NeuroD. Cotransfection of an RB expression plasmid had no significant effect on NeuroD dependent transcription, suggesting that RB does not functionally interact with NeuroD on the secretin promoter to increase transcription (Fig. 2C).

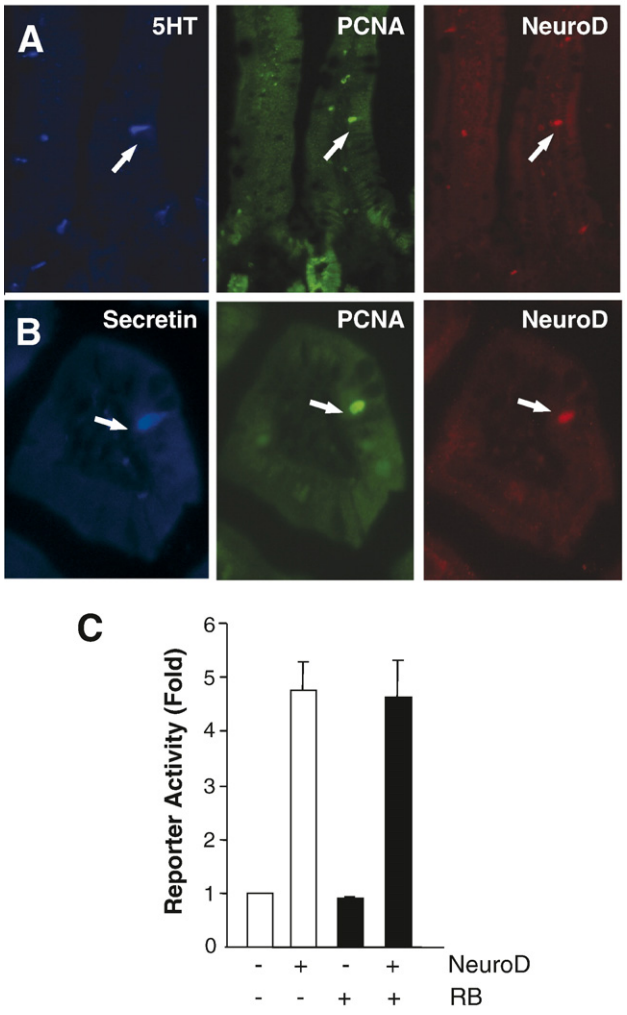

Fig. 2. NeuroD expression in proliferating endocrine cells. (A, B) Small intestine from *Ngn3-Cre;RB*$^{Flox/-}$ mice with triple immunofluorescent staining for PCNA (green), NeuroD (red), and either serotonin(5HT) or secretin (blue). Arrows denote identical cells in each channel. (C) Transient expression assays for NeuroD dependent transcription of a secretin luciferase reporter gene in C33A cells in the absence (white) and presence of transfected RB plasmid (black). Cells were cotransfected with indicated expression plasmids. Results were expressed as fold increase in luciferase activity ± SEM compared to the reporter alone.

RB regulates differentiation of serotonin and ghrelin cells in the antral stomach

We previously showed that most enteroendocrine cells in the antral stomach arose from Ngn3-expressing precursor cells by recombination-based cell lineage analysis (Schonhoff et al., 2004). To assess the role of RB in gastric endocrine cell differentiation, we stained stomach tissue from *Ngn3-Cre;RB* $^{Flox/-}$ mice and *Ngn3-Cre;RB*$^{Flox/-}$*;p107*$^{-/-}$ mice for different hormones normally expressed in the stomach. Examination of the antral stomach revealed a 2- to 3-fold increase in the total number of serotonin cells and a 3-fold decrease in the number of ghrelin-expressing cells (Figs. 3A, C, and D). In contrast, most other gastric endocrine cells in the stomach appeared in normal numbers with rare gastrin-expressing cells staining for PCNA (Figs. 3A, B, and Table 2).

Unlike the antral stomach, only a small fraction of enteroendocrine cells in the corpus of the stomach arise from Ngn3^{+} cells (Schonhoff et al., 2004). Thus most enteroendocrine cells in this region of the stomach were unlikely to undergo recombination and deletion of the floxed RB allele. As expected, we did not observe any differences in the enteroendocrine populations of the stomach body between wild type, *Ngn3-Cre;RB*$^{Flox/-}$ mice and *Ngn3-Cre;RB*$^{Flox/-}$*;p107*$^{-/-}$ mice (Fig. 3E and Table 2).

Serotonin cells are usually found near the base of the gastric glands in the murine antral stomach. Deletion of RB resulted in an increase in the number of serotonin cells with a shift in their distribution more towards the midportion or isthmus region rather than the base of the antral gland. A major fraction of serotonin cells stained for PCNA in RB mutants in contrast to antral serotonin cells of normal mice, which are quiescent (Figs. 4A–C and Table 2). Most PCNA-expressing serotonin cells were also identified in the isthmus of the glands, suggesting that they may represent relatively immature cells since this part of the gland contains proliferating progenitor cells as well as the pluripotent cells of the gastric epithelium (Table 2 and Fig. 4C). In addition, staining for caspase 3 expression showed no difference in the number of cells undergoing apoptosis (not shown), suggesting that the increase in serotonin cell number mostly results from increased cell proliferation.

To further characterize the increased numbers of antral serotonin cells that arise when RB is deleted in Ngn3^{+} cells, we crossed *Ngn3-Cre;RB*$^{Flox/-}$ mice into a *R26R* reporter strain, allowing us to identify all cells arising from Ngn3^{+} cells expressing Cre recombinase. Serotonin cells accounted for only a small fraction of the proliferating cells in the isthmus. However, most of the proliferating cells expressing β-galactosidase activity also expressed serotonin, indicating that conditional deletion of RB in Ngn3^{+} cells did not block their differentiation into neuroendocrine cells (Figs. 4D, E).

We previously showed that approximately 81% of chromogranin A^{+} enteroendocrine cells in the antral stomach of *Ngn3-Cre;R26R* mice expressed β-galactosidase (β-gal), indicating that they arose from Ngn3^{+} progenitors (Schonhoff et al., 2004). We unexpectedly found β-galactosidase activity in only 40% of serotonin cells, indicating that 60% of this lineage did not arise

Table 2
Cell cycle activity in gastric endocrine cells

Cell type	% PCNA$^+$ cells	
	RB$^{-/-}$	WT
Antrum		
Serotonin	56±3 (neck)	0±0
	16±4 (base)	
Gastrin	3±2 (base)	0±0
Somatostatin	0±0	0±0
Ghrelin	0±0	0±0
Body		
Serotonin	0±0	0±0
HDC	0±0	0±0
Somatostatin	0±0	0±0

Abbreviations: neck, neck/isthmus region of the gastric gland; base, base region of the gastric gland. The difference between serotonin cells and other gastric endocrine cells was significant with a P value<0.01. Three animals per genotype were analyzed. At least 500 cells were counted for each lineage. Results expressed as percent cells staining for PCNA±SEM.

from Ngn3$^+$ cells (Fig. 4F). This latter population does not appear to express chromogranin A (not shown). In *Ngn3-Cre; RB$^{Flox/-}$;R26R* mice, the fraction of antral serotonin cells expressing β-galactosidase rose to 75%, suggesting that there was a significant expansion of the serotonin cell population that arose from Ngn3$^+$ cells (Fig. 4G). In addition, all proliferating antral serotonin cells expressed β-galactosidase and were therefore descendants of Ngn3$^+$ cells (Figs. 4D, E). Thus in the absence of RB, serotonin cells that arise from Ngn3$^+$ cells appear to lose growth control at an early stage of differentiation and fail to migrate normally to the base of the glands.

To determine if cell cycle activity of antral serotonin cells versus ghrelin cells arose from differences in p130 expression, we examined these two endocrine cell types for p130 expression. The expression of p130 in the antral stomach has not been previously reported. Like the intestine, p130 is not expressed in all epithelial cells of the gastric glands. Most cells in middle–lower region and at the base of the glands show p130 immunostaining (Figs. 4H, K). Nearly all antral serotonin and ghrelin cells show expression of p130 (Figs. 4J, K), making it unlikely that p130 expression contributes to the differences in their response to the loss of RB.

Role of RB in pancreatic islet differentiation

Transient expression of Ngn3 in the fetal pancreas is a critical event for islet differentiation. Mice lacking this factor fail to develop pancreatic endocrine cells (Gradwohl et al., 2000). In addition, recombination based cell lineage analysis indicated that all islet cell types arise from Ngn3-expressing cells , indicating that the effects of Ngn3 were cell autonomous (Schonhoff et al., 2004). Unlike the intestine and stomach, the adult pancreas is a relatively quiescent tissue with a much slower turnover rate than the intestine or stomach. We have examined pancreatic islets in *Ngn3-Cre;RB$^{Flox/-}$* mice to determine whether RB is necessary at early stages of differentiation with the onset of Ngn3 expression for islet precursor cells to form mature islets. Islets in the RB/p107 mutants appeared indistinguishable from islets of normal mice with the central islet core comprised of insulin-expressing β cells (Fig. 5A). Non-β cells expressing somatostatin, glucagon, and PP were distributed around the periphery of islets of mutant mice and appear identical to their normal counterparts (Figs. 5B–D, PP not shown). The overall normal organization of the islets seen in the mutant mice indicates that RB and p107 are not required for normal islet morphogenesis. However, we observed a nearly 3-fold increase in proliferating cells stained with PCNA (from 17±5 versus 6±1 per 1000 μm^2 islet) in RB mutant mice (Figs. 5E, F), suggesting that RB family proteins may contribute to maintenance of quiescence in adult islets. Most of the proliferating cells were β cells. Loss of RB did not appear to produce islet hyperplasia with no apparent change in islet size. We identified small numbers islets in both mutant and wild type mice with one or two cells that stained for caspase 3 (Figs. 5G, H) suggesting that loss of Rb was not associated with increased apoptosis.

Discussion

Hormone-expressing cells represent a small subpopulation of the epithelial cells in a number of gastrointestinal organs including the stomach, small intestine, colon, and pancreas. The majority of gastric and intestinal enteroendocrine cells are generally found in regions distinct from the proliferating crypt compartment in the intestine and the isthmus region of gastric glands. The topographic separation of a proliferating compartment in these two organs from nondividing, differentiated cells implies that terminal differentiation of enteroendocrine cells is highly coordinated with cell cycle exit.

RB and related proteins function both to promote cell cycle exit and enhance expression of genes expressed in differentiating cells. Thus, RB could play an important role in coordinating terminal differentiation with cell cycle exit. Mice with RB and p107 or p130 gene deletion developed chronic hyperplasia in the distal small intestine, indicating RB family proteins restrict crypt cell proliferation (Haigis et al., 2006). We previously showed that generalized deletion of RB in the small intestine resulted in increased numbers of serotonin-expressing enteroendocrine cells remaining in the cell cycle. However, in our earlier study, the exact cell where RB was deleted was not identified or characterized.

In the present work, we have extended our earlier work by specifically deleting RB in a well-defined population of precursors to gastrointestinal endocrine cells expressing neurogenin3. We did not encounter the perinatal lethality seen with more widespread deletion of RB, allowing us to examine the role of RB in regulating differentiation of endocrine cells in the intestine, stomach, and pancreas during ongoing self-renewal of the GI tract in adult animals.

In normal mice, nearly all endocrine cells in the stomach and colon have exited the cell cycle, whereas small numbers of pancreatic islets and small intestinal substance P cells continue to cycle. Deletion of RB in mutant animals resulted in increased cell cycle activity in most tissues examined with the exception of the body of the stomach, where relatively few endocrine cells

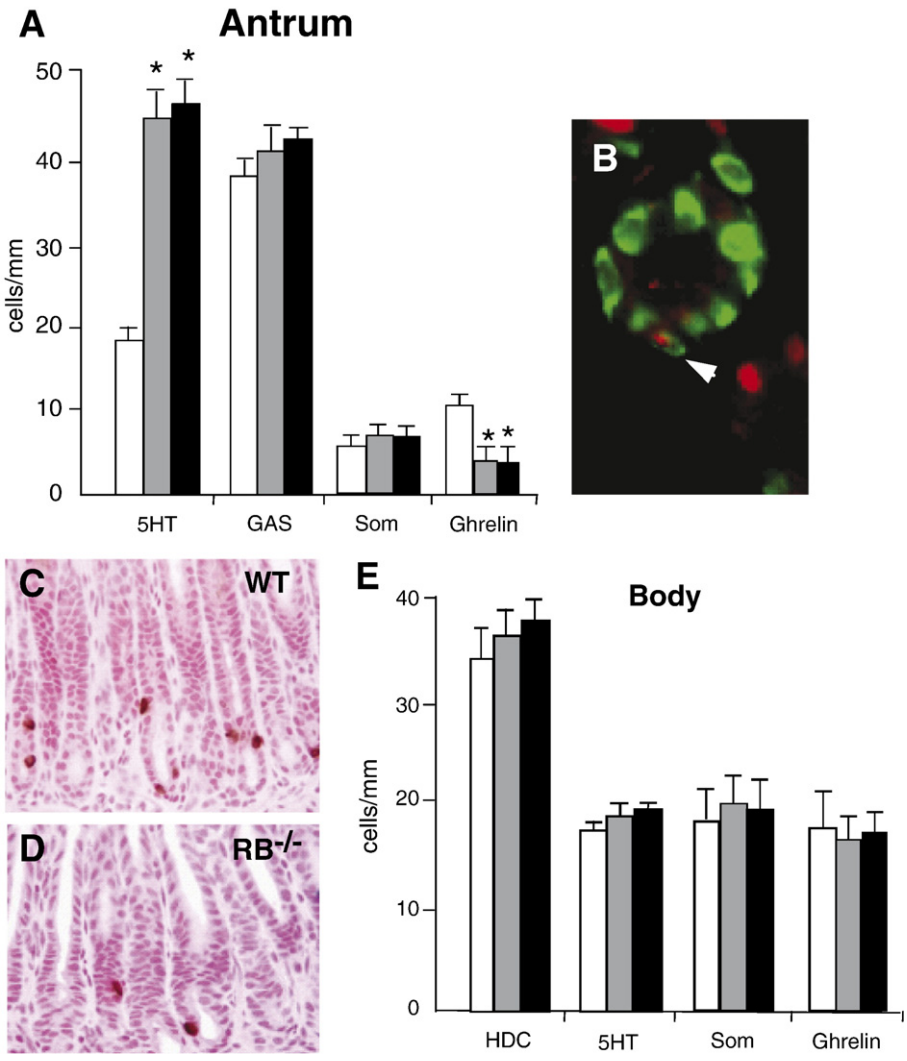

Fig. 3. Endocrine differentiation in the stomach of *Ngn3-Cre;RB*$^{Flox/-}$ mice. (A, E) Morphometric analysis of endocrine cells in the antrum (A) and body (E) stomach from wild type (white bars), *Ngn3-Cre;RB*$^{Flox/-}$ (gray bars), and *Ngn3-Cre;RB*$^{Flox/-}$*;p107*$^{-/-}$ mice (black bars). Results were expressed as mean number of stained cells ± SEM per mm length in longitudinal sections of the stomach. Four to six animals per group were examined. The asterisk (*) denotes $p < 0.01$ versus wild type. *P* values were determined by an unpaired, two tailed *t*-test. (B) Antral stomach from an adult *Ngn3-Cre;RB*$^{Flox/-}$ mouse with immunofluorescent staining for gastrin (green) and PCNA (red). (C, D) Immunostaining for ghrelin in the antral stomach of *Ngn3-Cre;RB*$^{Flox/-}$ mice (D) and wild type mice (C). Abbreviations: 5HT, serotonin; GAS, gastrin; Som, somatostatin; HDC, histidine decarboxylase.

arise from Ngn3^{+} cells and is not likely to have undergone recombination at the floxed RB allele. Deletion of RB alone appeared to be sufficient to induce cell proliferation in descendants of Ngn3^{+} cells. We did not observe increased cell proliferation in p107 mutant mice and RB/p107 double mutants were indistinguishable from the RB mutants. Thus it is unlikely that p107 partially compensates for the loss of RB.

Inactivation of RB family proteins in multiple cellular contexts has shown that RB-dependent cell cycle arrest is often linked to terminal differentiation. For secretin-expressing enteroendocrine cells, the failure to identify secretin in cells taking up tritiated thymidine as well as the restriction of secretin expression to villi has been interpreted to suggest that cell cycle arrest might be required for terminal differentiation (Inokuchi et al., 1985). Our findings suggest that cell cycle withdrawal is not obligatory for enteroendocrine cells to express their characteristic hormones in all organs examined. In addition to incomplete cell cycle exit, relatively subtle changes in differentiation resulted from the absence of RB, with increased numbers of hormone-expressing cells in intestinal crypts and in the isthmus of antral gastric glands, where relatively immature cell reside. This may indicate that RB is required for normal migration of differentiating cells away from the stem cell zone in these organs.

Serotonin cells in the intestine and stomach were most effected lineage in RB mutant mice and led us to consider whether the other endocrine cell types may not express the same RB family members as serotonin cells. Examination of RB family members in the small intestine showed RB expression in all epithelial cells, p107 expression confined to the crypt region, with p130 expression occurring largely in the villi (Haigis et al., 2006). The absence of RB expression in some enteroendocrine cells cannot explain the sensitivity of serotonin cells to RB loss since RB is present in all epithelial cells. Deletion of p107 alone did not produce a phenotype nor did it modify the RB null

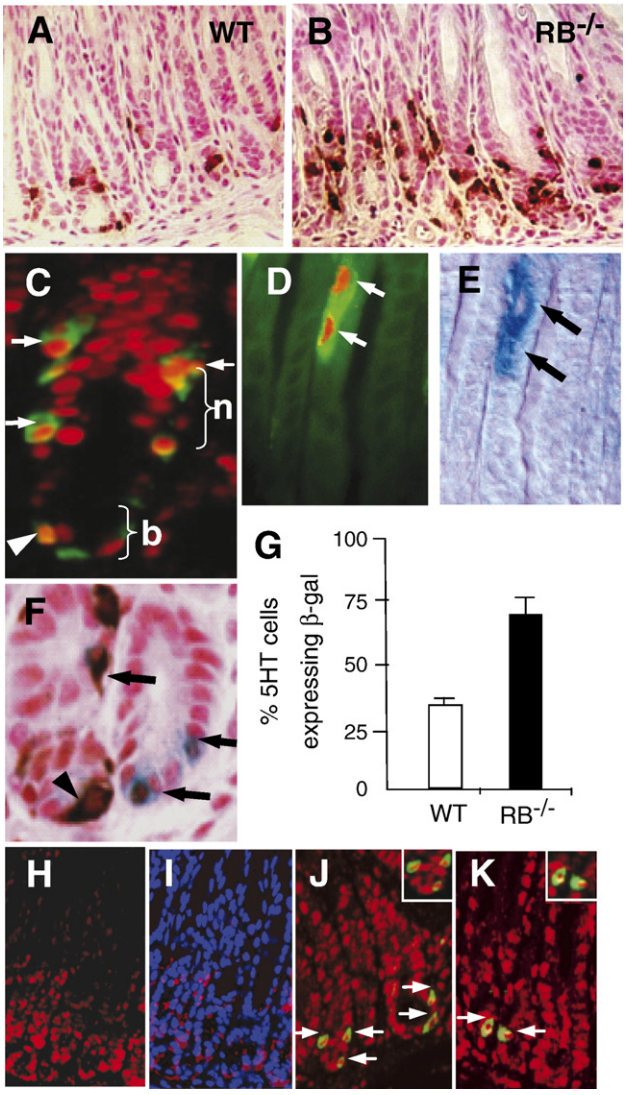

Fig. 4. Serotonin cells in the antral stomach of *Ngn3-Cre;RB*$^{Flox/-}$ mice. (A–B) Antral stomach of wild type mice (A) and *Ngn3-Cre;RB*$^{Flox/}$*;R26R*$^{-}$ (B) stained for serotonin. (C) Antral stomach of *Ngn3-Cre;RB*$^{Flox/-}$ mice with double immunofluorescent staining for serotonin (green) and PCNA (red). Arrows denote cells in the neck region (n) of the gastric gland expressing both PCNA and 5HT. Arrowhead denotes cells at base (b) of the gastric gland expressing both PCNA and 5HT. (D) Antral stomach of *Ngn3-Cre;RB*$^{Flox/-}$ mice with double immunofluorescent staining for 5HT (green) and PCNA (red). (E) Same section in panel D with x-gal staining for β-galactosidase activity. Arrow denotes cell expressing PCNA and 5HT with β-galactosidase activity. (F) Antral stomach of an *Ngn3-Cre;R26R* mouse with staining for β-galactosidase activity (blue) and serotonin (brown). Arrows denote cells expressing both β-gal and serotonin. Arrowhead denotes a serotonin cell without β-gal activity. (G) Percentage of serotonin cells (5HT) expressing β-galactosidase activity from wild type (white bars) and *Ngn3-Cre;RB*$^{Flox/-}$*;R26R* mice (black bars). (H, I) Confocal images of antral stomach from *Ngn3-Cre;RB*$^{Flox/-}$ mouse stained for p130, (H); or merged images of DAPI and p130 immunofluorescence, (I). (J, K) Double immunofluorescence for p130 (red) and serotonin (J, green) or ghrelin (K, green) in the antral stomach of an *Ngn3-Cre;RB*$^{Flox/-}$ mouse. Insets show several serotonin or ghrelin cells with nuclear staining for p130 at high magnification.

phenotype. It is unlikely that differences in p107 expression account for increased cell cycle activity of serotonin cells in RB mutants. In addition, relatively few enteroendocrine cells are localized to the crypt region where p107 is expressed. In the present work, we show that both serotonin and secretin cells express p130 in RB mutants, making it unlikely that the observed increase in serotonin cell proliferation results from the absence of functional compensation from p130. It is possible that deletion of RB results in expansion of serotonin-expressing PCNA^{+} enteroendocrine precursors that exit the cell cycle prior to differentiating into other cell lineages. However, our observation that other enteroendocrine cell types, in addition to serotonin cells, show increased cell cycle activity suggests that RB loss delays their maturation as well.

Cells that proliferate following the loss of RB continue to express NeuroD, a bHLH transcription factor we previously showed contributed to cell cycle withdrawal of differentiating enteroendocrine cells. This suggests that the effects of NeuroD on cell cycle activity are mediated in part by RB and agrees with earlier work from our group showing increased cell cycle

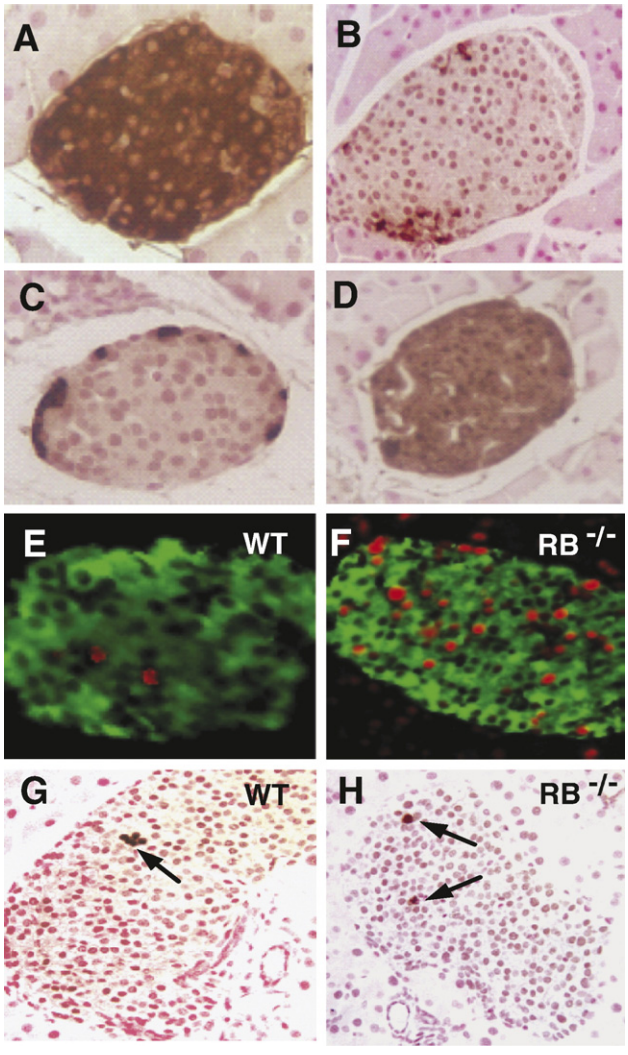

Fig. 5. Islet morphogenesis in *Ngn3-Cre;RB*$^{Flox/-}$ mice. (A–D) Pancreas from *Ngn3-Cre;RB*$^{Flox/-}$ mice with islets immunostained for insulin (A), somatostatin (B), glucagon (C) and chromogranin A (D). (E–F) Islets with double immunofluorescent staining for chromogranin A (green), and PCNA (red) in wild type (E) and *Ngn3-Cre;RB*$^{Flox/-}$ mice (F). (G, H) Islets from wild type (G) and *Ngn3-Cre;RB*$^{Flox/-}$ mice (H) show occasional cells stained for caspase 3, arrows.

activity and loss of $p21^{cip1/waf1}$ expression in NeuroD null mice and that NeuroD activates a p21 reporter gene (Mutoh et al., 1998). Thus, NeuroD may inhibit RB phosphorylation, keeping RB complexed with E2F to prevent expression of E2F target genes associated with the G1–S transition of the cell cycle. However, RB does not appear to function as a coactivator for NeuroD to potentiate transcription of the secretin gene as was described for the POMC gene (Batsche et al., 2005). The POMC and secretin genes are each regulated by composite enhancers. RB potentiates transcriptional activation of the POMC gene by NeuroD, serving as a scaffold for NeuroD to associate with another transcription factor, NGF1B, which is not involved in regulating the secretin gene.

Despite the significant increase in cell proliferation of several enteroendocrine lineages, the overall number of each endocrine cell type in the intestine was maintained. We saw no difference in caspase 3 staining in RB mutant mice to suggest a compensatory increase in apoptosis. In general, the ability to identify large numbers of apoptotic cells in the intestine is limited by the rapid clearance of apoptotic bodies within 1–2 h (Hall et al., 1994), making detection of enteroendocrine cells undergoing apoptosis extremely unlikely. Therefore, the likelihood of identifying changes in apoptosis of enteroendocrine cells may be extremely small since they comprise only a few percent of the epithelial cells in the intestine.

In the antral stomach where cells turn over more slowly, we observed 2- to 3-fold increase in the number of serotonin cells, unlike the intestine where the number of serotonin cells was maintained. In contrast, all other endocrine cells in the antral stomach, including the largest endocrine cell population, gastrin-expressing cells, showed normal cell density and distribution with rare proliferating cells. The sensitivity of serotonin cells in the antral stomach and small intestine to the loss of RB compared to other enteroendocrine cell lineages suggests additional, unappreciated heterogeneity between different enteroendocrine cell types. We also found increased numbers of serotonin-expressing cells in the isthmus region of the gastric gland, indicating that serotonin cell maturation may be also affected by loss of RB.

Moreover, in the antral stomach, the increased number of serotonin cells is accompanied by a significantly reduced number of ghrelin cells in RB mutant animals. One possible interpretation of this finding is that serotonin cells and ghrelin cells may arise from closely related progenitor cells in the antral stomach. Loss of RB may impose a positive effect on serotonin cell differentiation, which results in the expansion of serotonin progenitor cells. This may in turn deplete progenitor cells for other lineages, such as ghrelin cells. However, the reduction of ghrelin cells cannot account for the increase of serotonin cells.

Carcinoid tumors represent the predominant endocrine neoplasm of the alimentary tract in humans. Midgut carcinoids involving the stomach and small bowel frequently produce serotonin and occasionally substance P. Interestingly, serotonin cells appeared to be far more sensitive to the loss of RB than other enteroendocrine cell types. However, none of the mutant mice developed tumors, even after one year, indicating the RB loss alone is not sufficient for tumorigenesis. Rodents are unusually susceptible to developing type I gastric carcinoids with prolonged hypergastrinemia. These most commonly arise in enterochromaffin-like cells in the corpus of the stomach. However, in the present work, we did not target this cell population for deletion of RB since most endocrine cells in the stomach body arise independently of Ngn3. *RB* gene mutations are frequently observed in pulmonary small cell neuroendocrine carcinoma (SCNC), characterized by aggressive biological behavior and early metastasis (Meuwissen et al., 2003). Related small cell neuroendocrine tumors arise in the stomach and elsewhere in the GI tract and have been designated as poorly differentiated endocrine carcinoma (GI PDECs). Up to 60% of these tumors, as well as sporadic type 3 gastric carcinoids show loss of heterozygosity of the p16 gene and loss of expression of RB (Dacic et al., 2002; Pizzi et al., 2003). However, most of these tumors exhibit abnormalities in a number of oncogenic pathways, suggesting that in man, RB mutations alone may not be sufficient to develop GI endocrine tumors.

In summary, our work suggests that RB is important for enteroendocrine cells to undergo cell cycle arrest as they differentiate. However, enteroendocrine cells differentiate to express their characteristic hormones in the absence of functional RB or p107 proteins without exiting the cell cycle. The importance of RB for inducing cell cycle arrest of developing enteroendocrine cells is quite variable between different endocrine lineages and different gastrointestinal organs.

Methods

Conditional deletion of RB and P107 in Ngn3-expressing cells

To conditionally delete RB in Ngn3-expressing cells, *Ngn3-Cre* mice were crossed with $RB^{Flox/-}$ mice containing one RB null allele and one RB allele flanked by loxP sites. $RB^{Flox/-}$ mice were generated by crossing $FVB;129\text{-}Rb\text{-}1^{Tm2brn}$ strain (Vooijs et al., 1998) with *EIIa-Cre* mice, a general deletor strain from Jackson lab (*B6.FVB-Tg (EIIa-Cre)C5379Lmgd/J*). The $Ngn3\text{-}Cre;RB^{Flox/-}$ mice were further crossed to a p107 deletion background (Lee et al., 1996). In addition, $Ngn3\text{-}Cre;RB^{Flox/-}$ mice were crossed to homozygous R26R mice ($B6.129S4\text{-}Gt\ 26\ Sor^{tmSor}$) from Jackson laboratories (Soriano, 1999) to generate mice with both RB alleles deleted in $Ngn3^{+}$ cells in a R26R background.

Histology and immunohistochemistry

Tissue samples were fixed in 4% paraformaldehyde for 4–6 hours and processed for paraffin or frozen sections. Slides were incubated with the following antibodies for hormone staining: rabbit anti-chromogranin A at 1:1000 (ImmunoStar), rabbit anti-serotonin at 1:10,000 (ImmunoStar), rabbit anti-secretin at 1:500 (W.Chey, University of Rochester), rabbit anti-cholecystokinin at 1:8000 (Chemicon), rabbit anti-somatostatin at 1:3000 (R. Lechan, Tufts University), guinea pig anti-insulin at 1:500 (Sorin Biomedica), rabbit anti-glucagon at 1:300 (a gift from M Appel, University of Massachusetts), rabbit anti-somatostatin at 1:400 (a gift from R. Lechan, Tufts University), Guinea pig anti-PYY at 1:500 (a gift from G. Aponte, University of California, Berkley), rabbit anti-ghrelin at 1:25,000 (a gift from K. Kangawa, Osaka, Japan), guinea pig anti-histidine decarboxylase at 1:800 (Research Diagnostics), mouse antigastrin at 1:6000 (#E5, Cure, UCLA), rabbit anti-NeuroD at 1:1500 (Santa Cruz N19), anti-cleaved caspase-3 at 1:200 (Cell Signalling), and rabbit anti-p130 at 1:100 (Santa Cruz). Immunoperoxidase labeling was performed with a Vectastain ABC kit (Vector Labs) using 3,3′-diaminobenzidine precipitation for detection. Cell proliferation was assessed by immunostaining for PCNA and Ki67 using mouse anti-PCNA at 1:500 (Neomarkers) and mouse anti-Ki67 at 1:100 (BD Pharmingen). For immunofluorescent staining, antibodies were detected with Cy3-, FITC- or Alexa Fluor

350-conjugated secondary antibodies (Jackson Immunoresearch), or by tyramide amplification signal (TSA, Molecular Probes), which allowed co-localization studies using two rabbit primary antibodies.

Cell culture and transfection

The human cervical carcinoma cell line C33A (American Type Culture Collection) were cultured as previously described (Ray et al., 2003). The secretin–luciferase reporter construct consisted of sequences from −209 to +32 of the rat secretin gene (Wheeler et al., 1992) cloned upstream of the structural gene encoding firefly luciferase. The RB expression plasmid (pCMV-RB56) encoding amino acids 381 through 928, was described earlier (Smith et al., 1997). C33A cells were cotransfected with the reporter plasmids in the absence and/or presence of the expression plasmid for NeuroD or RB by the calcium phosphate method and assayed as previously described reference earlier work (Ray et al., 2003).

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